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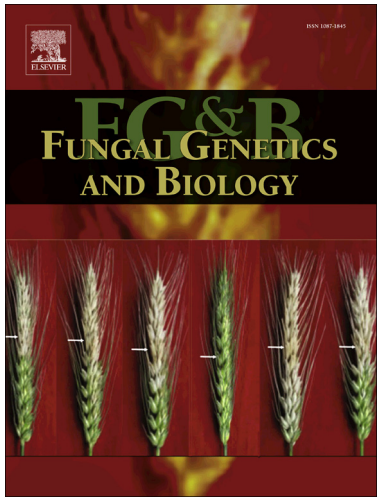

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Development and characterization of microsatellite markers for the oomyceta *Aphanomyces euteiches*

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Abstract

Aphanomyces euteiches Drechsler is a serious pathogen of leguminous crops that causes devastating root rot of pea worldwide. Given that *A. euteiches* is a diploid organism, robust, codominant markers are needed for population genetics studies. We have developed and screened a microsatellite-enriched small-insert genomic library for identification of *A. euteiches* SSR containing sequences. Fourteen out of the 48 primer pairs designed to amplify SSR, produced unambiguous polymorphic products in our test population of 94 isolates. The number of alleles at each locus ranged from one to four. The identification of new markers would enhance the ability to evaluate the genetic structure of *A. euteiches* populations, and pathogen evolution.

Keywords: *Aphanomyces*, genetic study, microsatellite, oomyceta, SSR

1. Introduction

Aphanomyces euteiches Drechsler is an oomycete pathogen of legumes, which causes a devastating root rot of pea (*Pisum sativum* L.) worldwide (Kraft and Pfleger, 2001). Initially considered as a pea root pathogen (Scott, 1961), *A.euteiches* was later reported to attack other legume species such as common bean, broad bean, faba bean, clover, and alfalfa (Pfender and Hagedorn, 1982; Greenhalgh and Merriman, 1985; Lamari and Bernier, 1985; Tofte et al., 1992; Burnett et al., 1994; Tivoli et al., 2006; Moussart et al., 2008). The only two efficient ways to control the disease are diverse crop rotations with non-host or suppressive crops and breeding for resistance. Both require knowledge of the genetic diversity and structure of *A.euteiches* populations. Thus, several molecular markers were developed, mainly dominant markers such as random amplified polymorphic DNA (RAPD) or amplified length polymorphism (AFLP). These techniques indicated the existence of different genetic subpopulations in North America, which differ by host of origin and host preference (Malvick et al., 1998; Grünwald and Hoheisel, 2006). All pea-infecting populations showed significant linkage disequilibrium between markers, supporting the hypothesis that selfing plays a major role in shaping their genetic structure (Grunwald and Hoheisel, 2006). The degree and spatial distribution of genotypic diversity within *A. euteiches* populations has also been studied in the USA, but reports have been contradictory. Indeed, using RAPD markers, Malvick and Percich (1998) found high genotypic diversity within fields, but no genotypic differentiation between populations from different locations (Wisconsin, Minnesota, Oregon). In a study of an alfalfa-infecting population in central USA (Illinois) using AFLP markers, Malvick et al. (2009) reported high levels of genotypic diversity at all scales but no geographical structure. Using AFLP markers, Grunwald and Hoheisel (2006) also found high genotypic diversity within field samples in Oregon and Washington states, but observed even higher genetic diversity at the regional scale and a significant genotypic differentiation between fields.

These differences in genetic structure of *A. euteiches* populations, and the fact that population genetic studies were carried out with dominant markers whereas *A. euteiches* is a diploid organism justify further investigation. The differ conclusions could be partly due to differences in the type and resolutive power of the genetic markers employed, and in particular to the ability to detect

heterozygotes; therefore, it is useful to explore new techniques to target variability in *A. euteiches* populations. Microsatellite (SSR) is a PCR-based molecular marker, which has many advantages, and has proved to be invaluable in many fields of biology, from genome mapping to forensics, and in population genetics (Dutech et al., 2007). SSR markers have been extensively used in genetic diversity studies (Struss and Plieske, 1998; Tenzer et al, 1999) because of their ubiquity, ease of scoring, co-dominance, reproducibility, assumed neutrality and high level of polymorphism (Maroof et al., 1994; Jarne and Lagoda, 1996). The work reported here had two main aims: first, to develop and screen a microsatellite-enriched small-insert genomic library for identification of *A. euteiches* SSR containing sequences, and second to identify the polymorphic SSR markers in a collection of 94 isolates.

2. Materials and methods

2.1 Microsatellite library development

Microsatellite markers were developed according to the procedure of Malausa et al. (2011) using next-generation sequencing and library enrichment. Eight isolates of *A. euteiches* from different locations (two from France and two from the US) and pathotype groups (pathotype I or pathotype III) were used to identify putative microsatellites sequences. Genomic DNA was extracted by using the CTAB method (Abdelnoor et al., 1995). Construction, emulsion PCR and 454 GS-FLX titanium pyrosequencing of the microsatellite-enriched DNA libraries were carried out by Genoscreen (Lille, France). Enriched libraries were constructed using eight microsatellite probes ($(AG)_{10}$, $(AC)_{10}$, $(AAC)_8$, $(AGG)_8$, $(ACG)_8$, $(AAG)_8$, $(ACAT)_6$, $(ATCT)_6$).

2.2 DNA isolation and SSR amplification

A total of 94 different *A. euteiches* isolates, which included 66 French isolates (Fr1 to Fr66. All isolates belong to pathotype I) sampled from six different locations, 24 American isolates (13 isolates belong to pathotype I (Us01 to Us10, Us20, Us21, Us24), and 11 isolates belongs to pathotype III (Us11 to Us19, Us22, Us23)) sampled from three different locations, and four referent isolates (Rb84 (sampled from a pea field), Ae109, MF1 and NF1 (sampled from alfalfa fields)) were used

(**Table 1**). Total genomic DNA was extracted from these 94 isolates using the CTAB method (Abdelnoor et al., 1995). Each PCR reaction in simplex consisted of 10 µL reaction volumes containing 20 ng template DNA, 2µL of 5x PCR buffer [20 mM Tris-HCl ph 8.8, 10 mM KCl, 10 mM $(Nh4)_2SO4$, 2 mM MgSO4, 0.1% Triton X-100], 0.2 mM each dNTP, 1 µM of each forward and reverse primer, 0.25 µL of 10 µM fluorescent-labeled M13 primer (VIC; Applied biosystem), and 1 unit of Taq polymerase. Volumes were adjusted to 10 µL with sterile distilled water and amplification reactions were conducted on a S1000 Thermal Cycler (Bio-Rad). Cycling conditions included an initial denaturation at 95 °C for 5 min, followed by 20 cycles of denaturation at 95 °C for 60 s, annealing at 58 °C for 60 s and extension at 72 °C for 90 s, followed by 20 cycles of denaturation at 95 °C for 60 s, annealing at 53 °C for 60 s and extension at 72 °C for 90 s and a final extension at 72 °C for 10 min. PCR products were then diluted 1:40 in sterile water and 3 µL of this dilution was mixed with 0.05 µL of GeneScan 500 LIZ Size Standard (Applied Biosystem) and 5 µL of formamide (Applied Biosystem). Analyses of PCR products were conducted on an ABI Prism_ 3130xl sequencer (Applied Biosystem). Allele size determinations were achieved using the GeneMapper software v3.7 (Applied Biosystem) by manual identification of the peaks and validation of absence or non-valid chromatogram. Allele sizes were determined by the automatic calling and binning module of GeneMapper with manual examination of irregular results.

2.3 Statistical analysis an evaluation of polymorphism

The allelic diversity or polymorphism information content (PIC) was measured for each selected polymorphic SSR as described by Botstein et al. (1980). *Aphanomyces euteiches* isolates were clustered on the basis of their genetic relatedness using multivariate analyses. A factorial correspondence analysis (FCA) was performed for the overall data set using GENETIX 4.05.2 (Belkhir et al., 2004).

3. Results

3.1 SSR polymorphism

A total of 48 primer pairs were designed and tested. Fourteen markers showed polymorphisms and therefore could be used as SSR markers (**Table 2**). Thirty percent of the SSRs were polymorphic, and the number of alleles per locus was 2-4 (average 2.5). The number of genotypes detected per locus ranged from 2-4 (average 2.9), and the percentage of heterozygous genotypes ranged from 0-24.5%. The PIC value of each marker varied between 0.101-0.511 (average 0.389) (**Table 2**). According to the criteria proposed by Botstein et al. (1980), only one (7.2%) of the SSR markers was highly informative (PIC > 0.5), 11 (78.6%) were reasonably informative (0.25 < PIC < 0.5), and 14.3% slightly informative (PIC < 0.25).

3.2 Diversity and cluster analysis

The genetic structure of the American and French *A.euteiches* populations was investigated using the 14 polymorphic SSRs markers. Pea-infecting isolates were collected from four major pea cropping areas in the US where pea and/or alfalfa were grown and from seven fields (differing in crop rotation) from seven major pea cropping areas in France. Based on the polymorphic SSR markers, results indicated low genetic diversity among the different locations in France and the US, with only eight multilocus genotypes observed within the 94 *A. euteiches* isolates tested (**Fig. 1**). The greatest variation occurred within countries, with a total estimated genetic diversity (F_{ST}) of 0.436 for *A. euteiches* between American and French populations. Factorial correspondence analysis (FCA) analyses showed that the genetic structure of *A. euteiches* populations was dependant on the country of origin but not on the pathotype. The first axis explained 57% of the structure, whereas the second axis explained only 13%. Based on pathotype groups, these results suggest that *A. euteiches* pea populations in France are a unique, unstructured population. However, among French populations, two different clusters were observed: one corresponding to isolates sampled from fields where other legume species (vetch, alfalfa, or faba bean) were cultivated during previous growing seasons, and another corresponding to isolates sampled from fields where pea was the only legume crop species.

4. Discussion

The development and screening of a microsatellite-enriched small-insert genomic library identified fourteen highly polymorphic SSR markers from a total of 48 SSR sequences. These markers produced unambiguous polymorphic products in our test population of 94 isolates. The PIC value of each marker varied between 0.101-0.511, and 85.7% of the primers were reasonably informative (Botstein et al., 1980).

The number of polymorphic SSR observed (30%) is quite low, but not surprising. By comparison, Akamatsu *et al*. (2007), by generating and screening a plasmid library of *A. euteiches* genomic DNA identified only eleven SSRs markers on the sixty-nine primers pairs screened for allelic variation (10% if considering all the markers tested). Moreover, their study showed that 50% of these SSRs markers indicated two polymorphic bands whereas in our study polymorphic SSRs indicated 2-5 different loci. A limited number of polymorphic microsatellite loci isolated from genomic libraries are reported for peculiar biological and genomic traits of fungi (Dutech et al., 2007). First, plant pathogens, which are the most extensively studied of fungal species, have demographic and reproductive traits promoting low genetic diversity. Crop or human pathogen have often experienced bottlenecks through geographic introduction (Milgroom et al., 1992; Engelbrecht et al., 2004; Rivas et al., 2004) or host shifts (Mackenzie et al., 2001; Paraskevis et al., 2003; Tobler et al., 2003), which can drastically reduce intraspecific genetic diversity. Furthermore, some specific life history traits of fungal pathogens, such as frequent asexual reproduction and recurrent bottlenecks in epidemic cycles, associated with low winter survival and/or selective sweeps following new virulence attributes, are also likely to result in a low level of genetic diversity (Goodwin et al., 1994; Hovmoller et al., 2002; Guérin and Le Cam, 2004). Second, several papers have examined the nature and abundance of microsatellites in published partial or complete fungal genomes, and these have appeared less abundant in these fungal genomes than in other organisms (Lim et al., 2004; Dutech et al., 2007; Stewart et al., 2011).

The use of SSR markers in our study indicated that *A. euteiches* populations seemed to be less diverse that was indicated in past studies (Malvick et al., 1998; Grünwald and Hoheisel, 2006;

Malvick et al., 2009). Indeed, genetic diversity was low among locations in France and among locations in the US, with only eight multilocus genotypes observed within the 94 *A. euteiches* isolates tested. Factorial correspondence analysis indicated that the genetic structure of *A. euteiches* populations was dependant on the country of origin but not on the pathotype (Fst = 0.856). Within French populations, two different clusters were observed, one corresponding to isolates sampled from fields where other legume species (vetch, alfalfa, or faba bean) were cultivated previously, and another corresponding to isolates sampled from fields where pea crops were grown as the only legume species. Thus, for the American *A. euteiches* populations and some French isolates, these results suggest that cultural practices and more particularly the frequency of different legumes species play an important role in the population structure. Indeed, this homothallic pathogen is polyphagous and was also reported to attack other legume species (common bean, broad bean, faba bean, clover, and alfalfa) (Moussart et al., 2008). As sexual recombination and gene flow remained low in population evolutionary processes, there may be a possible role for these host plants in the population structure.

To conclude, these SSR markers prove useful in characterizing genetic variation within and among *A. euteiches* populations. These markers will be helpful to the community to better understand how this soil-borne disease could evolve in their population diversity and how cultural practices could or not modify this diversity (Hernandez-Delgado, 2009). More particularly, information gathered from population genetics studies may improve models of disease epidemics and forecasting, enhance the evaluation of risks to established plant cultivars, or assist in targeting control measures. *Aphanomyces euteiches* was reported to attack other legume species (Levenfors, 2004; Moussart et al., 2008). However, infection of several host plants is a prevalent process in agrosystems, leading to change in epidemic process and pathogenicity (Woolhouse et al., 2001). Adaptation event of *A. euteiches* to host plant resistance has already been observed in alfalfa, as isolates overcoming genetic resistance to race 1 of *A. euteiches* have been identified (Grau et al., 1991). Consequently, the impact of alternative host on plant pathogen adaptation processes must be taken into account for the durable management and of resistance cultivars. For instance, incorporating knowledge of a pathogen's population structure into breeding for disease resistance may provide insight into the potential long-term and global effectiveness of resistant breeding lines. The importance of sexual reproduction for the maintenance of

polymorphism or for the adaptive dynamic of the species Links between wild and agricultural pathosystems exist, but in many cases an insufficient knowledge regarding the diversity and biology of the pathogens have led to wrong conclusions (Duan *et al*., 2003). By using SSRs markers with highest allelic diversity, we could well address questions of paternity or clonal structure. We can determine if populations deviated from Hardy-Weinberg equilibrium, if there is more or less heterozygote within populations, and if there is outcrossing within populations (McDonald & Linde, 2002; Montarry *et al*., 2010). Finally, as with many soil borne pathogens *A. euteiches* has limited means of dispersal, thus gene flow is thought to be limited (McDonald & Linde 2002). The use of these SSRs markers will be helpful to study the role of anthropic activities on dispersal processes and on *A. euteiches* migration between neighboring fields.

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Figure caption

Fig. 1: Factorial correspondence analysis (FCA) performed on 94 *A. euteiches* isolates from France and US. Each point represents one or several isolates. Three genetically distinct clusters were highlighted: the first groups together all individuals from most of the different locations, the referent isolate Rb84 and four American isolates, the second groups together all individuals from two fields where other legume species than pea were cultivated, and the third groups together individuals from the different American locations, and the three referent isolates Ae109, NC1, MF1 (isolates sampled on alfalfa).

Table 1: *Aphanomyces euteiches* isolates used in this study

Code	Effective	Country	Plant Origin	Pathotype
Fr1to Fr66	66	France	Pea	Pathotype I
Us1to Us10, Us20, Us21, Us24	13	US	Pea	Pathotype I
Us11to Us19, Us22, Us23	11	US	Pea	Pathotype III
Rb84	1	France	Pea	Pathotype I
Ae109	1	US	Alfalfa	Pathotype III
NF1	1	US	Alfalfa	Pathotype III
MF1	1	US	Alfalfa	Pathotype III

Table2: Details of locus, primer sequence, *Tm*, Motif, no. of alleles, allele size, % of heterozygotes, and PIC value of different SSR markers used to evaluate genetic diversity within *Aphanomyces euteiches* isolates

Locus	Forward primer	Reverse primer	*Tm* (°C)	Motif	No. of alleles	Allele size	% heterozygotes	PIC
Ae04	TATTGTCTTGCTGGATTGGAA	ACGATCTCCTGAATGCCG	58	$(ACG)_5$	2	90	5.3	0.101
Ae12	CATCTTTCGTCTTCGGATCG	GCCTCTGACGTCCAAATTGA	58	$(TCG)_8$	3	90	0.0	0.496
Ae13	CGATAGTCCTAGCGTCTTCCTT	GATATCAAATGCATTCAACCG	58	$(TCG)_5$	2	91	0.0	0.382
Ae17	TCTTGATGCCGTTCATGTTC	CATTTTGCGCCGTTCTATTT	58	$(TCG)_5$	2	127	5.3	0.444
Ae23	GCGAAGGCCAGGTACTAAAG	TGAATTCAAGAATAATCGGAAAG	58	$(AC)_6$	2	197	5.3	0.444
Ae26	TTTCAAGGCCAGGAGAAAGA	TTCATCAACGGCAAACACAT	58	$(AGA)_5$	2	229	25.7	0.382
Ae32	GACGTGTTTGAAACCAACGA	ATTTGCTCCCAACAAACGAC	58	$(TCG)_5$	2	113	0.0	0.173
Ae34	TGAAAATGATCCTCGAATCCA	AAATCTTCCATCAACGCCAC	58	$(GAC)_6$	2	121	5.3	0.444
Ae36	CCATGGATGCAGTCATCAAC	CTTGCACCCATGCTCTACAA	58	$(CCAG)_5$	3	164	24.5	0.375
Ae37	GTACTGGCACCTTCTCCTCG	CGAAATAGGGGCTTTGTCTG	58	$(CTC)_6$	4	138	5.3	0.511
Ae44	TCAACTCTGGGTAGGACATTCG	TGATCTTGTAGAGATGTCGTATTTCG	58	$(TC)_7$	4	140	0.0	0.406
Ae45	AACGGCAAACAACAGAACG	AAGTGCCGAGGTAGACAACG	58	$(GA)_6$	2	146	5.3	0.444
Ae54	GTAGCAAAGTGACCGTCGTG	CGATGGTCTAGGTGCTAACG	58	$(AG)_8$	3	145	17.0	0.474
Ae63	GCCGAAGCAGTTGAAGAAGT	CCTCGGCTGTTTCAGCAT	58	$(AAG)_6$	2	97	24.5	0.370

Fig. 1: Factorial correspondence analysis (FCA) performed on 90 *A. euteiches* isolates from France (grey circle) and US (Black Square) populations, and four referent isolates (white diamond). Each point represents one or several isolates. Three genetically distinct clusters were highlighted: the first groups together all individuals from most of the different locations, the referent isolate Rb84 and four American isolates, the second groups together all individuals from two fields where other legume species than pea were cultivated, and the third groups together individuals from the different American locations, and the three referent isolates Ae109, NC1, MF1 (isolates sampled on alfalfa).